Review

RNA modulation, repair and remodeling by splice switching oligonucleotides[★]

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Targeting splicing by antisense oligonucleotides allows RNA modifications that are not possible with RNA interference or other antisense techniques that destine the RNA for destruction. By changing the ratio of naturally occurring splice variants the expression of mRNA is modulated. By preventing the use of an aberrant splice site created by a mutation and enforcing re-selection of correct splice sites the RNA is repaired. Antisense induced skipping of the exon that carries a nonsense mutation remodels the mRNA and restores the reading frame of the defective protein. All of the above approaches have clinical applications. Modulation of splice variants is particularly important since close to 60% of all genes code for alternatively spliced pre-mRNA.

Interest in mRNA as a target for drug therapy has intensified recently with discovery of RNA interference (RNAi) and subsequent attempts to use small interfering RNAs (siRNAs) as potential therapeutics (Agami, 2002). RNA's appeal is easy to appreciate: the nucleotide sequence provides an opportunity to design sequence-specific and therefore

gene-specific drugs. For the most part, research and development of RNA based therapeutics have focused on downregulation of expression of target genes. This review will focus on an oligonucleotide based technology, invented in this laboratory (Dominski & Kole, 1993; Kole & Dominski, 1997) that generates modified or repaired mRNAs (see a series of articles in *Journal of Clinical Investigation*, vol. 112, issues 3 & 4), an outcome that is not possible with RNAi and other antisense based techniques (Crooke, 2001). RNA repair and modulation is accomplished by application of antisense oligonucleotides, termed splice-switching oligonucleotides, (SSOs), which modify alternative splicing patterns of pre-mRNA. The importance of this approach is underscored by the recent realization that the frequency of alternative splicing is higher than previously anticipated, with most studies reporting alternative splice forms in up to 60% of human genes (Modrek & Lee, 2002). This mini review presents recent use of SSOs to modulate splicing of targets that illustrate the range of this technology's applications.

RNA MODULATION: Bcl-x AND CANCER

Bcl-x, like other members of the *bcl-2* family of genes is involved in apoptosis. Its two primary splice forms, bcl-xL and bcl-xS, are generated by alternative splicing of bcl-x intron 2 (Cory & Adams, 2002 and references quoted therein), *via* the use of a common 3′ splice site and two alternative 5′ splice sites. Bcl-xL, the longer splice variant, is anti-apoptotic. Moreover, high levels of bcl-xL have been correlated with resistance to chemotherapy (Liu *et al.*, 1999). The shorter variant, bcl-xS, which uses a 5′ splice site farther upstream from the 3′ splice site than bcl-xL, has been shown to have anti-apoptotic properties (Minn *et al.*, 1996). SSOs targeted against the bcl-xL 5′ splice site were shown to shift splicing towards production of bcl-xS in the lung

epithelial cancer cell line A549 (Taylor *et al.*, 1999). Although the splice shift alone was insufficient to induce apoptosis, it enhanced the apoptotic effects of chemotherapeutics administered with the oligonucleotide. In contrast, prostate cancer PC3 cells, were sensitive to SSO treatment in the absence of anti-cancer drugs (Mercatante *et al.*, 2001). The cell sensitivity was correlated with the level of *bcl-x* expression or more accurately with the absolute amounts of bcl-xS generated by the anti-bcl-xL SSO. In addition, the SSO treatment sensitized the cells to chemotherapeutics and radiation and interestingly led to cell death in a cell line that was resistant to doxorubicin, a common anti-cancer drug (Mercatante *et al.*, 2002).

Bcl-x is a particularly useful target for SSOs because the two splice variants have opposing functions. Thus, a shift in splicing simultaneously reduces the level of anti-apoptotic Bcl-xL and increases the level of pro-apoptotic Bcl-xL, enhancing the SSOs effect. The results also suggest that expression of possibly thousands of alternatively spliced genes can be manipulated by SSOs either as research tools in investigations of gene function, or possibly as drugs when targeted to disease associated genes, following the example of *bcl-x* (Mercatante & Kole, 2000).

RNA REPAIR: β-THALASSEMIA

β-Thalassemia is a blood disease resulting from reduced or absent production of functional β-globin, a subunit of hemoglobin. Severe cases of thalassemia, if untreated, result in early death. Current treatment consists of frequent transfusions combined with iron chelation therapy. Inability to produce hemoglobin is caused by mutations in the β-globin gene, affecting various stages of β-globin biosynthesis. While over 200 mutations lead to disease, several common forms of thalassemia are due to mutations in the β-globin gene introns that create alternative splice

sites and lead to aberrant splicing of pre-mRNA. The result is inclusion of the intron fragment, which creates a nonsense codon in the β-globin coding sequence, preventing translation of the protein (Olivieri, 1999). This laboratory has used splice-switching oligonucleotides to target aberrant splice sites and induce a shift from aberrant to correct splicing, restoring expression of hemoglobin. Sequence-specific correction of splicing was achieved in model cell lines and subsequently by *ex vivo* treatment of erythroid progenitors cells from thalassemia patients (reviewed in Sazani *et al.*, 2002a). In these studies, the SSOs were delivered by cationic lipid delivery agents or by temporary, mechanical damage to cell membranes (Lacerra *et al.*, 2000). Free uptake of SSOs by the cultured erythroid cells was also accomplished using oligonucleotides with a neutral, morpholino backbone. High levels of correct RNA and hemoglobin expression were achieved after several days of treatment (Suwanmanee *et al.*, 2002a; 2002b).

Like most other pharmacological agents, SSOs effects are relatively short term due to their degradation and excretion. Thus, the possibility of long-term repair of β-globin pre-mRNA has also been investigated. The sequence antisense to the aberrant splice sites in β-globin pre-mRNA was cloned into a U7 snRNA gene, and the modified anti-β-globin U7 snRNA expressed from its own promoter served as a means to deliver antisense sequences to erythroid cell nuclei. Previous work relied on transfection of appropriate plasmids. Recently the modified U7 gene was incorporated into lentiviral vectors (Vacek *et al.*, 2003). Treatment of cells, both model cell lines and thalassemic patients erythroid progenitor, resulted in significant upregulation of β-globin mRNA and functional protein expression. Although the level of expression was not as high as in the SSO treated cells, the effects were permanent in the cell lines and lasted as long as the primary erythroid cells could be maintained in culture (Vacek *et*

al., 2003). Antisense RNA delivery *via* lentiviral vectors is especially appealing in chronic diseases such as β-thalassemia that require lifelong correction of splicing.

One advantage of RNA repair, especially for the gene that is as tightly developmentally controlled as β-globin is that the target gene is expressed in its natural environment and therefore cannot be over- or inappropriately expressed. As mentioned in the introduction, RNA repair allows up-regulation or re-expression of a defective gene, a result that cannot be achieved by RNAi or standard antisense approaches.

RNA REMODELING: DUCHENNE MUSCULAR DYSTROPHY

Duchenne muscular dystrophy (DMD) is an X-linked disorder resulting from nonsense or frameshift point mutations in the dystrophin gene that prevent translation of functional dystrophin protein (Matsuo, 1996). Inability to produce dystrophin leads to progressive muscle wasting and early death. Interestingly, large internal deletions, preserve the translational reading frame, resulting in shortened but partially functional dystrophin, lead to a milder form of the disorder, Becker muscular dystrophy (BMD). Several groups have investigated antisense therapy in *mdx* mouse models of DMD, in which a nonsense mutation in exon 23 disrupts the reading frame of the dystrophin gene. In earlier work SSOs were targeted to 5′ and 3′ splice sites to induce skipping of exon 23, thus resulting in a shortened but in-frame dystrophin protein: in essence, antisense therapy was designed to create partially functional BMD-like protein, reducing the DMD phenotype (Wilton *et al.*, 1999). More recently, antisense oligonucleotides injected into limbs of *mdx* mice with nonsense dystrophin mutations were shown to partially restore dystrophin production and induce dystrophin protein localization in the muscle sarcolemma. Importantly, the

mice regained partial function in the injected muscle (Gebski *et al.*, 2003; Lu *et al.*, 2003).

Targeting of DMD presents an interesting and novel use of SSO technology. Although the dystrophin gene is not alternatively spliced, and DMD does not arise from splicing mutations, SSOs treatment can be used to repair the RNA and restore the function of a defective gene.

ANTISENSE DEVELOPMENT: POSITIVE READ-OUT ASSAY

Although antisense oligonucleotides are getting close to being accepted as therapeutics and are undergoing numerous clinical trials (http://www.clinicaltrials.gov/) their delivery and cellular uptake still need improvement. An assay that provides clear, sequence specific and sensitive read-out would be very useful in further investigations of the improved oligonucleotides. Modification of splicing by SSOs, which results in generation of a new splice variant on a null or low background, provides such an assay. This assay was devised and exploited in this laboratory to analyze in cell culture and *in vivo* the antisense effects of several novel oligonucleotide chemistries.

The assay utilizes an enhanced green fluorescent protein (EGFP) the coding sequence of which is interrupted by a defective intron from human β-globin gene. The intron carries a thalassemic mutation that creates an aberrant 5′ splice site and activates an additional 3′ splice site upstream. The resultant aberrant splicing prevents production of correct EGFP mRNA and protein (Sazani *et al.*, 2001). For *in vitro* and *in vivo* testing, this construct was either stably transfected into HeLa cells or expressed in a transgenic mouse model. In the absence of oligonucleotide treatment virtually no EGFP mRNA was present and very little or no background fluorescence was detected in cultured cells or mouse tissues. These systems test sequence-

specific antisense activity; the generated fluorescence is directly proportional to the effectiveness of the antisense molecules in splice shifting. A positive readout confirms that the antisense molecule was able to cross the plasma and nuclear membranes, bind to its target pre-mRNA in the nucleus in a sequence specific manner and interfere with the normal functioning of the spliceosome. The results can be quantified at the mRNA or protein level, by fluorescence-activated cell sorting (FACS) analysis of cultured cells or microscopy of sectioned mouse tissue. The assay allows comparison of effectiveness of oligonucleotide analog chemistries, delivery agents and different delivery approaches.

Using this assay, antisense properties of peptide nucleic acid (PNA), morpholino, 2′-*O*-methoxyethoxy (2′-O-MOE) and 2′-*O*-methyl (2′-O-Me) were investigated. Interestingly, with 'free uptake' of the oligonucleotides into cultured HeLa cells, with no special delivery methods or agents, antisense efficiency seemed to be related to a net backbone charge. The neutral PNA and morpholino analogs performed better than their negative counterparts (2′-O-MOE and 2′-O-Me). The addition of four lysine residues to PNA analogs increased their efficacy presumably due to a positive charge conferred by the basic amino acids (Sazani *et al.*, 2001).

Consistent with *in vitro* cell line data, the PNA-4-lysine chemistry out-performed all other oligonucleotides tested *in vivo,* exhibiting significant fluorescence in the liver, kidney, heart and lung tissues of the EGFP mouse. Unexpectedly, 2′-O-MOE treated animals also showed intense response to treatment with detectable fluorescence in several tissues while the neutral morpholino oligonucleotide analog showed little response in all tissues tested (Sazani *et al.*, 2002b). Lack of morpholino effects is likely a consequence of rapid renal clearance of such molecules (Agrawal *et al.*, 1995). Recently the EGFP-based splicing assay was also used for cell culture testing of oligonucleotide based

modifications that increased binding of these compounds to their target. No effect on splice shifting was observed (Sazani *et al.*, 2003). An earlier version of the assay, based on luciferase read-out (Kang *et al.*, 1998) was also used to study morpholino oligonucleotides conjugated to peptides some of which were found to improve cellular and nuclear uptake of the conjugates (Moulton *et al.*, 2003).

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